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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this research is to study the role of the antagonistic relationship between two opposing growth signals, IGF and TGFß, in mammary turmorigenesis. We set out to test a hypothesis that the induction of IGFBP-3 by TGF-ß in stromal fibroblasts is a mechanism by which TGF-ß regulates the growth of breast epithelial cells. To do this, a model cell culture system was established in which the effects of TGF-ß through IGFBP-3 could be studied. Our results show that recombinant IGFBP-3 is able to block IGF-induced growth of breast cancer cells, however the effects of secreted IGFBP-3 from fibroblast media are unclear. Growth inhibition does occur, but the presence of other molecules in this system cloud IGFBP-3's contribution. Additionally, we set out to define the mechanisms by which TGF-ß induces IGFBP-3 in MRC-9 cells. We determined that the gene is regulated by TGF-ß at the level of transcription, and not through mRNA stability. Analysis of the promoter (-1800 bp) for TGF-ß regulatory elements, however, showed that TGF-b did not significantly induce IGFBP-3 promoter activity in MRC-9 fibroblasts. Therefore, the location of the TGF-b transciptional reglulatory elements in the IGFBP-3 gene remain unknown.

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Anita Bat 09/29/99

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INTRODUCTION

The overall goal of the proposed project was to examine the roles and interactions of the TGF-ß and IGF growth signals in breast tumorigenesis. TGF-ß is a multifunctional peptide that plays a role in a wide variety of normal cellular functions including the regulation of proliferation, differentiation, extracellular matrix deposition, cell adhesion and migration (1). However, the effects of TGF-ß can be highly cell-type and even cell-state specific. In normal mammary epithelial cells, TGF-ß has been shown to be a potent inhibitor of growth through paracrine and autocrine pathways. TGF-ß acts through a direct mechanism as an inhibitor of proliferation by inducing the expression of the cyclin/cdk inhibitors such as p15, p21, and p27 in many cell types. Therefore, lesions to TGF-ß signaling pathways that disrupt the negative growth regulation of breast epithelia may contribute to mammary carcinogenesis and represent an obligatory step in neoplastic progression of breast epithelia. Indeed, loss of TGF-ß responsiveness of MCF7 breast cancer cells has been shown to correlate with a loss of expression in these cells of the TGF-ß signaling receptors (2). Loss of such autocrine control by TGF-ß represents an opportunity for malignant epithelia to increase proliferation in response to other positive growth factors, like IGF, and hormones, like estrogen.

Available evidence strongly suggests that an another aspect of TGF-ß-induced growth inhibition of breast cancer epithelia may involve countering the growth stimulatory effects of IGF by inducing the secretion of IGFBP-3. IGFBP' s are a family of molecules which sequester and prevent IGF' s from binding and transducing mitogenic signals through the IGF receptors. As interactions between breast epithelial and stromal cells have been implicated as being important in the genesis and proliferation of breast cancer, we wanted to test whether fibroblasts affect the growth of breast epithelia by secreting IGFBP-3 in response to TGF-ß. Such a phenomena, if perturbed, could contribute to mammary carcinogenesis.

Therefore, we initiated a study aimed at determining the roles IGF, IGFBP-3, and TGF-ß have in breast tumorigenesis. In the past twelve months, our work has been focused on two main areas: analysis of the interactions between breast epithelial and fibroblast cells mediated by IGF, IGFBP-3 and TGF-ß in cell culture and characterization of the molecular mechanism by which the expression of IGFBP-3 is regulated by TGF-ß. These studies were proposed as Technical Objectives 1 & 2 in the original proposal.

BODY

A. Optimization of Model Cell Culture System

As discussed above, TGF-ß may repress the growth of mammary epithelial cells through its induction of IGFBP-3 from stromal fibroblasts under normal and malignant conditions (3). This activity of TGF-ß may represent an indispensable mechanism by which the actions of growth stimulatory factors such as IGF are inhibited at the time when the cell cycle progression of epithelial cells is blocked by the TGF-ß signal. Furthermore, the effects of TGF-ß on the activity of IGF through its function of IGFBP-3 may represent an important aspect of interactions between mammary epithelial cells and stromal fibroblasts during normal mammary development as well as carcinogenesis. While several lines of evidence supported this notion in the literature (4,5,6), during the first year of this research we tested this hypothesis in a more direct fashion by establishing a model cell culture system using two MCF7 lines and MRC-9 fibroblasts as model epithelial and stromal cells. Over the past year, time was mainly spent trying to optimize the established cell culture system in an effort to surely conclude whether the secreted IGFBP-3 from the fibroblasts is adversely affecting the growth of breast cancer cells.

Indeed, the results of initial studies showed that conditioned media from MRC-9 cells containing IGFBP-3 induced by TGF- ß could function to block IGF mediated proliferation in MCF7 cells (Figures 2 & 3). Thus, it appeared that the conditioned media from the TGF-ß did have an anti-proliferative effect, which may be mediated by the secreted IGFBP-3. However, TGF- ß was still present in the conditioned media from MRC-9 cells and could be responsible for the observed growth inhibitory effect on the MCF7 cells. Although the basal proliferation of MCF7 cells increased when the conditioned media was added, the relative growth inhibition between the control -/+ TGF- ß and conditioned media -/+ TGF-ß treated MCF7 cells were not significantly different at 64% and 50%, respectively. Over the past year, we tried to precipitate out the residual TGF-ß with specific antibodies, in an effort to show that part if not all of the observed growth inhibition was due to IGFBP-3 in the conditioned media experiments. Interestingly, anti-TGF-ß antibodies had a growth stimulatory effect when added to the conditioned media, an unexpected and confusing result. Overall, it appears that there are many growth factors present in the conditioned media besides TGF-ß, which makes interpretation of this data difficult. On the other hand, if the growth inhibitory response is even partially due to IGFBP-3, we proposed in the last update using a neutralizing antibody against IGFBP-3 to block the observed growth inhibitory response. We hypothesized that it was possible that the proliferation would increase even more with the addition of such a neutralizing antibody. Unfortunately, anti-IGFBP-3 antibodies were ineffective in our cell culture system to show this.

One explanation of the data is that, in contrast to the recombinant IGFBP-3, the secreted IGFBP-3 from MRC-9 cells in inactive. It is possible that MCR-9 cells not only secrete IGFBP-3 but also the protease that inactivates it. Since the initiation of this study, there have been reports in the literature identifying specific IGFBP proteases being present in the media of some cell types. These proteases inactivate IGFBPs, preventing them from binding and sequestering IGFs. Hence, such proteases represent another level of regulation of IGFs and IGFBPs that had not been considered until recently. In fact, there is evidence to suggest that such proteases are up-regulated by certain cancers to overcome the anti-proliferative effects of IGFBPs.

Recommendations:

The above mentioned experimental challenges and limitations have led us to abandon further study of the cell culture system to analyze the role of secreted IGFBP-3. We feel the only way to truly elucidate the role of IGFBP-3 in TGF-ß mediated negative growth regulation of breast epithelial cells would be to engineer a transgenic mouse that either overexpressed or deleted IGFBP-3 in a breast tissue specific manner. Such an endeavor, however, is beyond the scope of this study and will not be attempted.

B. Promoter Analysis of IGFBP-3

As shown in Figures 1 and 4, we have found that TGF-ß potently induces IGFBP-3 transcript and protein in MRC-9 cells. And, as Figure 5 shows, this induction of mRNA is not dependent on new protein synthesis (which is blocked by cyclohexamide), suggesting TGF-ß' s effect on the IGFBP-3 gene is direct. As the IGFBP-3 gene has been shown to be transcriptionally up-regulated by the tumor suppressor gen p53, regulation of IGFBP-3 expression at the transcriptional level may be a general mechanism through which the expression of IGFBP-3 can be induced by anti-proliferative signals. To determine whether the increase in IGFBP-3 mRNA is due to transcriptional activation by TGF-ß, nuclei from MRC-9 cells treated with or without TGF-b were harvested and a nuclear transcription run-off assay was performed (Figure 6A). As a control, total RNA from those treated cells was also saved and an RNAse protection assay was performed (Figure 6B). This assay allows for the direct measurement of RNA transcripts that have already been initiated at the time of cell harvesting. The result of the run-off assay showed that TGF-ß did alter the rate of IGFBP-3 gene transcription after 6 and 24 hours of treatment by approximately 1.8-3 fold. Thus, the increase in IGFBP-3 mRNA after 6 and 24 hours of TGF-ß treatment is due to an increase in transcription. Consistent with this conclusion, is that there does not appear to be a component of mRNA stability as actinomycin D treatment, following TGF-ß treatment of MRC-9 cells for 8 hours, had little to no effect on the half-life of the IGFBP-3 message compared to control (data not shown).

The first step in defining the mechanism by which TGF-ß transcriptionally regulates IGFBP-3 gene expression is to determine the regions of the IGFBP-3 promoter responsible for transcriptional activation. To this end, we obtained approximately 1.8kb of the IGFBP-3 promoter upstream of the transcription start site, and cloned it into a reporter vector which drives the expression of a luciferase reporter gene (pGL2-Basic). Figure 7 diagrams the 5' deletions which were made of this promoter, in an effort to narrow down a TGF-ß responsive region. These constructs were transiently transfected into MRC-9 cells and TGF-ß induced luciferase activity was assayed. Unfortunately, after many attempts and conditions, no significant induction by TGF-ß was observed for any of the IGFBP-3 promoter constructs (Figure 8). In contrast, as a positive control, the common TGF-ß responsive reporter, p3TP-lux, gave a 5 fold induction of luciferase activity. From this data, it appears that the TGF-ß responsive region for MRC-9 cells is not located within –1.8 kb of the promoter. Furthermore, sequences we obtained from another lab that corresponded to regions in introns 1 and 2 were not responsive. To test whether this promoter region would be active in another cell type, these promoter regions were transfected into primary human aortic smooth muscle cells. These cells were also shown to respond to TGF-b by inducing IGFBP-3 protein and mRNA. The result of these experiments yielded similar results as the MRC-9 cells, showing insignificant induction of the promoter by TGF-ß (data not shown).

To pursue this further, we performed a genomic library screen on a human placental library in the effort to obtain more sequence from introns 1-4, as many genes are known to contain enhancer

elements in intronic sequences, as well as in sequences many kb away from their promoters. We screened the library using 800bp of IGFBP-3 coding sequence, and obtained genomic sequence that appeared to correlate with introns 1-3. These sequences have proven too difficult to isolate and amplify by PCR as they are high in G-C content, and have thus not been analyzed for TGF-b response in MRC-9 cells. Using promoter sequence as a probe, we were also not able to isolate sequences of the IGFBP-3 gene beyond 1.8kb to analyze. This screen yielded only sequences we already had. Potentially, our library has been amplified too many times and has lost representation of all sequences as a result.

Recommendations:

Even though IGFBP-3 transcript and protein are strongly induced by TGF-ß in MRC-9 cells, the promoter region is not. One explanation is that the TGF-b regulatory element used in MRC-9 cells is not present within –1.8kb of the promoter and is located elsewhere, possibly in distant sequences outside of the IGFBP-3 gene. If this is the case, much effort and time would be required to make another genomic library, which is not our expertise, and undertake more genomic library screens and cloning experiments to locate this elusive regulatory region.

Future Work:

Over the next twelve months, we feel that we can gain a better understanding of TGF-ß's complex role in breast cancer progression by refocusing the statement of work and specific aims of this study. Specifically, we have begun to investigate the role of the recently identified Smad molecules in mediating TGF-ß signaling.

The Smads are a family of proteins consisting of at least eight members. Smad2 and Smad3 are downstream effectors of TGF-ß. In response to TGF-ß, Smad2 and Smad3 are phosphorylated by the type I receptor kinase and translocate to the nucleus in a complex with Smad4. While in the nucleus they act as sequence specific transcription factors at the consensus site: GTCTAGAC. Several genes to date are known to be regulated by Smads at similar sequences including PAI-1, p3TP-Lux, COL7A1, JunB, and c-Jun. Smads are also thought to synergize or act in combination with other transcription factors such as the Vitamin D receptor, AP-1, Sp-1, p300/CBP, and MuE3 (TFE3) to activate transcription.

A role for Smads as tumor suppressors has been suggested by the observation that Smad2 and Smad4 are commonly deleted in a variety of cancers including the majority of human pancreatic, certain colon cancers, and some breast cancers. Thus, in light of their role as transcription factors, Smads may regulate genes which are critical for TGF-ß mediated growth inhibition. Consistent with this hypothesis, mouse embryo fibroblasts and lymphoid cells derived from Smad3 deficient mice have been found to be impaired in TGF-ß mediated growth inhibition. Although there is only one report implicating Smads in regulating the known TGF-ß growth inhibition pathway through p21 promoter induction, it is likely that other yet identified genes important for cell growth will be found to be linked to transcriptional regulation by Smads.

While TGFβ induces cell cycle arrest and Smads are known tumor suppressors, late stage breast cancers produce large amounts of TGFβ and are refractory to its growth inhibitory effects. Since TGF β can also stimulate cell invasion of breast cancer cells, TGFβ may initially act to suppress tumor formation but promote metastasis in later stages of disease. Indeed, recent evidence suggests that the signal transduction cascade may be required for breast cancer metastasis to the bone. A reduction in osteolytic metastasis is observed when MDA-MB-231 cells over-expressing dominant negative type II receptor are injected into nude mice (7). The loss of TGFβ signal transduction results in fewer tumors

with reduced numbers of osteoclasts and increased animal lifespan. Additionally, over-expression of constitutively active TGFβ type I receptor to potentiate the signal cascade results in increased metastasis with a corresponding decrease in survival (7).

Two genes potentially involved in the ability of TGFβ to promote bone metastasis are parathyroid hormone related protein (PTHrP) and interleukin 11 (IL-ll), which are both expressed by metastatic MDA-MB-231 cells (8,9). PTHrP increases bone resorption (10) and increases osteolytic metastasis when over-expressed in breast cancer cells (11). TGFβ treatment increases PTHrP expression *in vitro* (12), and over-expression of TGFβ type I receptor *in vivo* results in increased PTHrP (7). IL-11 promotes bone resorption *in vitro* by increasing osteoclast formation (13), and secretion of the cytokine is increased in osteolytic bone metastasis induced in nude mice (14). TGFβ can activate the IL-ll promoter through a mechanism involving AP1 family members (15) and Smad3 (data not shown). Recombinant human interleukin-ll is being used in clinical trials in breast cancer patients to prevent thrombocytopenia induced by chemotherapy (16-17), so elucidation of the potential role of interleukin-11 in osteolytic metastasis is of immediate clinical importance.

We would like to test the hypothesis that Smad molecules are required for the ability of breast cancer cells to metastasize to bone. While initial experiments supported by the final year of funding on this grant will evaluate the loss of Smad3 signaling on the ability of breast cancer cells to adhere to bone *in vitro*, further *in vivo* experiments will extend beyond the duration of the grant. The creation of Smad3 null mice by our laboratory provides a valuable tool to determine if TGFβ mediated signal transduction in the bone is required for breast cancer metastasis. An *in vitro* model measuring adhesion of breast cancer cells similar to that of van der Pluijm *et. al.* (18) will be established using the bone of neonatal mice. Tails of the mice will be dissected to obtain trabecular bone and frozen for cryostat sectioning. Tissue sections will be incubated with cells isolated from mammary cancer induced in wild type and Smad3 null mice through use of the polycyclic aromatic hydrocarbon 7,12-dimethylbenzanthracene. After incubation to allow for cell attachment to the bone, the sections will be washed, fixed, and mounted for cell number counts using light microscopy after staining with hematoxylin and eosin. If cell isolation becomes problematic, MDA-MB-231 cells will be transfected with pRK5F Smad3ΔC which lacks the site for phosphorylation by type I receptor and will act as a dominant negative. The ability of breast cancer cells deficient in Smad3 to adhere to wild type bone as compared to those expressing Smad3 will indicate whether TGFβ signal transduction in the breast cancer cells alone is important for adherence.

KEY RESEARCH ACCOMPLISHMENTS

- Recombinant IGFBP-3 effectively blocks IGF-1 stimulated proliferation of MCF7 cells in culture, and represents a potential key mediator of TGF-b mediated growth inhibition of breast epithelial cells.
- TGF-b strongly induces IGFBP-3 protein and mRNA in MRC9 fibroblasts
- The rapid induction of IGFBP-3 mRNA by TGF-b does not require new protein synthesis, and is a result of transcription and not mRNA stability
- The TGF-b cis-regulatory elements do not appear to reside within –1.8kb of the IGFBP-3 proximal promoter

REPORTABLE OUTCOMES

- Elissa Rougier-Chapman (Principal Investigator) obtained Master of Science degree in Molecular Cancer Biology from Duke University, September, 1999.

- Publications:

1) Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li Jian-Ming, Wang X-F. 1999. Smad3/Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by Transforming Growth Factor-ß. Mol. Cell. Bio. 19(3): 1821-1830.

2) Liberati NT, Datto MB, Frederick JP, Shen X, Wong C, Rougier-Chapman EM, Wang X-F. 1999. Smads bind directly to the Jun family of AP-1 transcription factors. Proc. Natl. Acad. Sci. 96: 4844-4849.

CONCLUSIONS

Transforming growth factor ß (TGF-ß) is a potent growth inhibitor of normal breast epithelia. Evidence is accumulating that TGF-ß activity is critical to maintaining the negative paracrine and autocrine regulation of breast epithelial growth. Therefore, defects in the TGF-ß signaling pathways may result in uncontrolled growth of mammary epithelial cells and consequently contribute to carcinogenesis of the breast. Available evidence suggests that TGF-ß is able to indirectly mediate its growth inhibitory effects on breast epithelia by inducing the secretion of insulin-like growth factor binding protein 3 (IGFBP-3). Our goal was to test a hypothesis that the induction of IGFBP-3 by TGF-b in stromal fibroblasts is a mechanism by which TGF-b regulates the growth of breast epithelial cells. We established a model cell culture system of MCF7 cells and MRC-9 fibroblasts and showed that in culture recombinant IGFBP-3 is able to block IGF-induced growth of breast cancer cells, however the effects of secreted IGFBP-3 from fibroblast media are unclear. Growth inhibition does occur, but the presence of other molecules in this system cloud IGFBP-3' s contribution. In fact, we believe the secreted IGFBP-3 may be inactive, as a result of specific IGFBP proteases that could be present in the MRC9 media. The implication of this, with so many variables to take into account, is that the cell culture system becomes ineffective at defining the relative contributions of the different factors in paracrine regulation of breast cancer cell growth. As mentioned above, one would have to design a transgenic mouse model to specifically "knock-out" or constitutively overexpress IGFBP-3 in the mammary tissue of mice to determine the true significance of this molecule in regulation of epithelial cell growth.

Besides the cell culture studies, we were investigating the molecular mechanism by which the expression of IGFBP-3 is regulated by TGF-ß in MRC-9 fibroblasts. We determined that the gene is regulated by TGF-ß at the level of transcription, and not through mRNA stability. Analysis of the promoter (-1800 bp) for TGF-ß regulatory elements, however, showed that TGF-b did not significantly induce IGFBP-3 promoter activity in MRC-9 fibroblasts. Additional genomic library screens also did not yield sequences containing TGF-ß regulatory elements. Therefore, the location of the TGF-ß transciptional reglulatory elements in the IGFBP-3 gene remain unknown.

Overview of recommended changes to work:

Since all of the specific aims of the original research proposal have been addressed, experiments for the final year of funding will shift focus to elucidate the involvement of TGF-ß in later stages of breast cancer. Specifically, we will test the hypothesis that Smad 3 is required for the ability of breast cancer cells to metastasize to bone. Understanding this pathway may result in the identification of molecular targets useful for drug design in the prevention and treatment of a currently incurable disease state.

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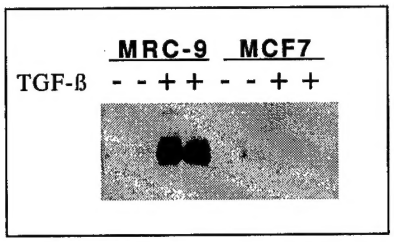

Figure 1: Western blot analysis of IGFBP3 secreted protein induction in conditioned media fromMRC9 and MCF7 (a breast cancer epithelial cells) following 48 hours of TGFβ treatment.

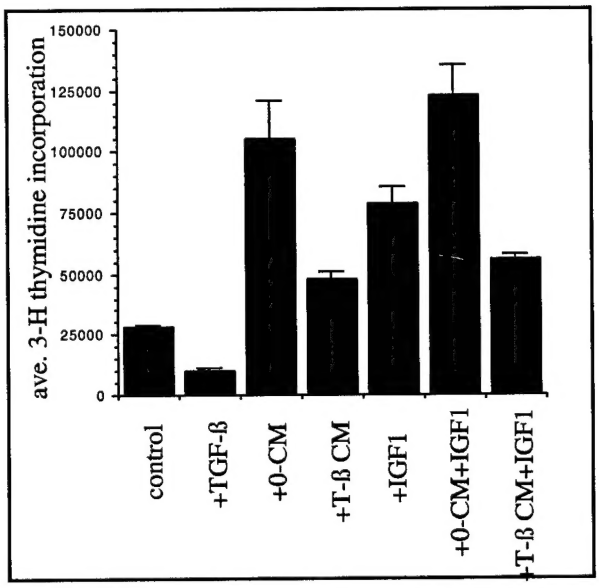

Figure 2: MCF-7/PT3 cell culture paracrine test of cell growth after treatment with TGF-ß, IGF1, or conditioned media (CM) from MRC-9 fibroblasts treated with or without TGF-ß.

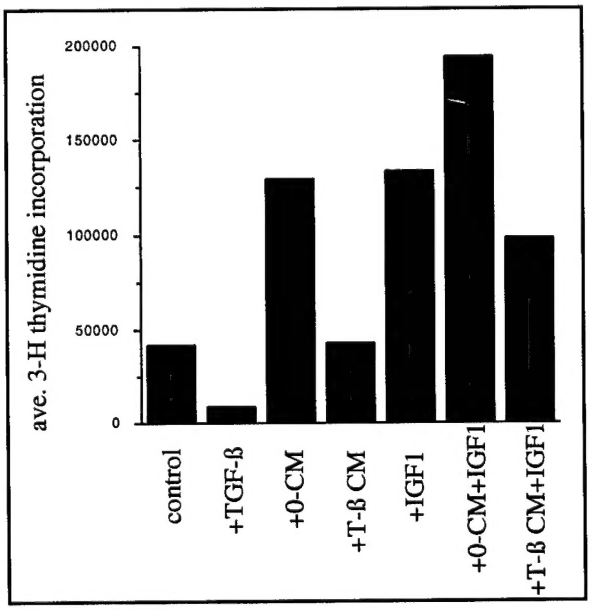

Figure 3: MCF-7/TEX cell culture paracrine test of cell growth after treatment with TGF-ß, IGF1, or conditioned media (CM) from MRC-9 fibroblasts treated with or without TGF-ß.

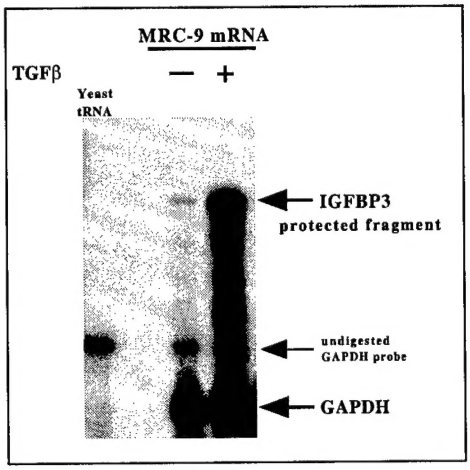

Figure 4: RNase protection analysis of IGFBP3 mRNA induction in MRC9 fibroblasts following 26 hours of TGFβ treatment.

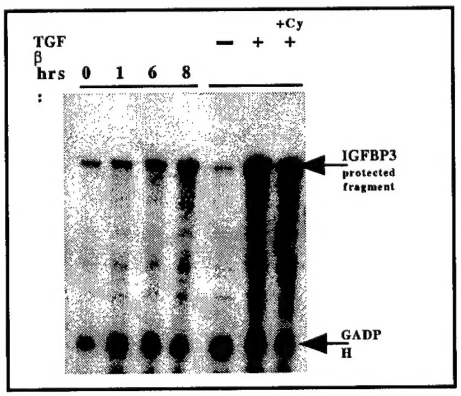

Figure 5: RNase protection analysis of IGFBP3 mRNA induction in MRC9 cells following 0,1,6, and 8 hours of TGFβ treatment and after 8 hours of TGFβ addition in the presence of cycloheximide.

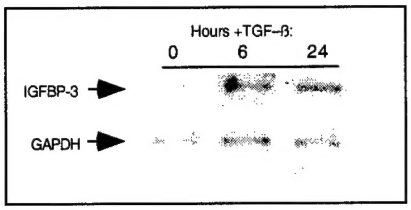

Figure 6A: Transcription runoff assay of nuclei from MRC-9 fibroblasts treated with or without TGF-ß for the indicated times. The 32P labelled mRNA transcripts were hybridized to IGFBP-3 and GAPDH cDNAs, which were immobilized to nitrocellulose.

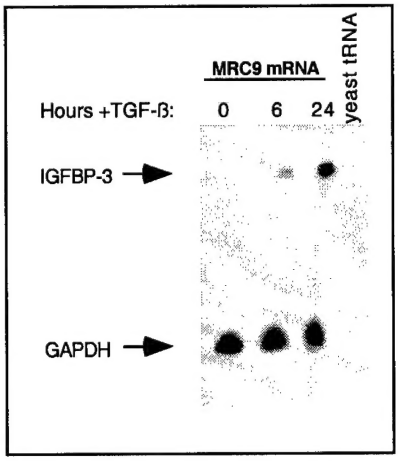

Figure 6B: RNAse protection assay of total RNA harvested from the TGF-ß treated MRC-9 cells used in Figure 6A.

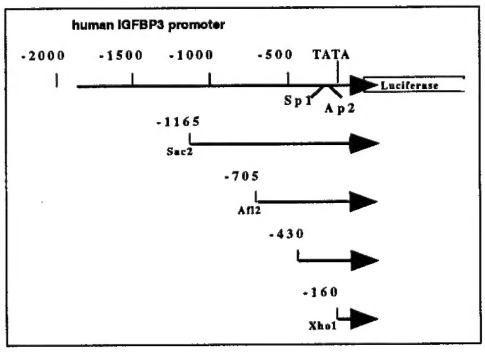

Figure 7: Diagram of the IGFBP3 promoter fragment obtained from Dr. D. Powell (-1800 bps) and the deletion constructs created from the Powell promoter. All promoter constructs were subcloned in front of the luciferase reporter gene.

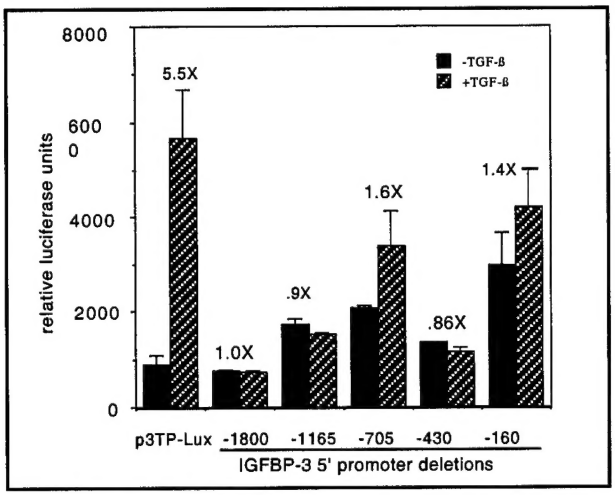

Figure 8: Luciferase assay of MRC-9 cells transfected with the various 5' promoter deletions of the proximal IGFBP-3 promoter and treated with or without 100pM TGF-ß. As a positive control, p3TP-Lux was also included. Fold inductions are shown above each set of bar graphs.